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Mixed PEGylated surfactant modifying system decrease the accelerated blood clearance phenomenon of nanoemulsions in rats

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ABSTRACT

The accelerated blood clearance (ABC) phenomenon which is induced by repeated injection of poly (ethylene glycol) (PEG)-coated colloidal carriers gives clinical challenge to the promising drug delivery system. It is necessary to decrease this unexpected immunological response. A novel 4-arm poly (ethylene glycol-5000)$_4$-cholesteryl methyl amide (4-arm PEG_{5000}-CHMA) has been synthesized. The structure of 4-arm PEG_{5000}-CHMA was confirmed by IR and ^{1}H-NMR spectrum. The pharmacokinetics of the tocopheryl nicotinate (TN)-loaded nanoemulsions modified with 4-arm PEG_{5000}-CHMA or/and 1, 2-distearoyl-Sn-glycero-3-phosphoethanolamine-n-[methoxy(poly-ethyleneglycol)-2000] ($mPEG_{2000}$-DSPE) have been studied. Furthermore, the ABC phenomenon has been detailed investigated in rats by TN-loaded nanoemulsions modified with 4-arm PEG_{5000}-CHMA and $mPEG_{2000}$-DSPE (CPNE). The plasma levels of TN and anti-PEG IgM antibody were determined by HPLC and ELISA, respectively. The circulation time of the CPNEs were comparable to the $mPEG_{2000}$-DSPE coated nanoemulsions. Moreover, the ABC phenomenon can be decreased by CPNEs. This study designs a method to decrease the ABC phenomenon and develops a clinical promising nanoemulsion for therapeutic or imaging purpose.

1. Introduction

The accelerated blood clearance (ABC) phenomenon, an unexpected immunological response that repeated administrated of PEGylated liposomes in the same animal with certain intervals will lead to a reduction in the circulation time and an increase in hepatic and splenic accumulation, has been extensively observed and is attracting more attention [1–5]. B cells, which are in the margin of spleen endowed with

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potent effector functions, are extremely required for the induction of ABC phenomenon through producing IgM. The antibody induced by the first dose of nanoparticles which acts as an TI-2 antigen in the induction phase, can combine with the second dose of PEGylated nanoparticles to activate the complement, leading to the opsonization of C3 and then accelerate the clearance of the second dose by the uptake of mononuclear phagocyte system (MPS) in the effectuation phase [6–8].

This phenomenon can also be induced by many other PEG-conjugated substances/PEGylated nanocarriers, such as PEGylated nanoemulsions [9], PEGylated nanoparticles [10], PEGylated micelles [11], PEGylated proteins [12], and decrease therapeutic efficacy [13]. Therefore, avoiding or alleviating the ABC phenomenon is very important and valuable not only for scientific research but also for the applicability of drug delivery system.

Over the past few decades, some alternative polymers have been reported to circumvent the ABC phenomenon. In the year of 2010, Tsutomu Ishihara demonstrated that nanoparticles covered with poly(N-vinyl-2-pyrrolidone), poly(4-acryloylmorpholine), or poly(N,N-dimethylacrylamide) did not induce the ABC phenomenon. When focused on the pharmacokinetics of these nanoparticles, the clearance value of PEGylated nanoparticle (3.7 ml/h/kg) is significantly smaller than those of nanoparticles covered with poly(N-vinyl-2-pyrrolidone) (25.7 ml/h/kg), poly(4-acryloylmorpholine) (19.0 ml/h/kg), or poly(N,N-dimethylacrylamide) (14.1 ml/h/kg), respectively [14]. In the year of 2013, Amr S. Abu Lila proposed that the ABC phenomenon was not induced by liposome modified with polyglycerol (PG) upon repeated injection procedure in rats. However, the $AUC_{0-24\,h}$ (303.7 ± 209.2 %Dose·h/ml) of PEGylated liposome is larger than PG coated liposomes (152.4 ± 29 %Dose·h/ml) [15]. Obviously, one of the drawbacks in using these proposed alternative polymers is the loss of the long circulation character of nanoparticles, which is essential for clinical applications [16].

We are here to discuss a method with mixed PEGylated surfactant modifying system in the field of attenuating the ABC phenomenon. In our works, we covered the nanoemulsions with linear PEG ($mPEG_{2000}$-DSPE) and branched PEG (4-arm PEG_{5000}-CHMA) in different ratio. This method can keep the long-circulating character and decrease the immunogenicity of PEGylated nanocarriers.

The novel branched PEG material, 4-arm PEG_{5000}-CHMA, has been synthesized and it features a complex structure comprising four long PEG chains. Each four chains have a total of 128 OCH_2CH_2 subunits in average value. The branched PEG material has a structural characteristic of cross-linking. Interestingly, the ABC phenomenon would be alleviated by nanoemulsions modified with $mPEG_{2000}$-DSPE and PEG_{5000}-CHMA. Moreover, these CPNEs also showed a longer half-life character, resulting in a potential accumulation in the inflammatory and tumorous lesion by the enhanced permeability and retention effect [17]. From the results of ELISA, we proposed that CPNEs disturb the secretion of antibody. In addition, 4-arm PEG_{5000}-CHMA decreases the following antibody binding. Then, the ABC phenomenon was decreased. Finally, we find a method to decrease the ABC phenomenon.

2. Materials and methods

2.1. Materials

4-arm PEG5000 (JenKem Technology, China); cholesterol chloroformate (CHMA, Acros Organics, USA); triethylamine (TEA, Tianjin Bodi Chemistry, China); dichloromethane (DCM, Zhengxin high-tech research institute, China); tocopheryl nicotinate (TN, Northeast Pharmaceutical Group, China); medium-chain triglycerides (MCT, Beiya Medicated Oil, China); injectable soybean lecithin S75 (S75, Lipoid GmbH, Germany); $mPEG_{2000}$-DSPE (Genzyme Corporation, USA); 50% (*m/v*) glucose solution (Shandong Yuwang Industry, China).

2.2. Synthesis of 4-arm PEG_{5000}-CHMA

The 4-arm PEG_{5000}-CHMA was synthesized using 4-arm PEG5000 and cholesterol CHMA, as starting materials and TEA as acid binding agent. The related accompanying reactions are outlined in Fig. 1. Solution A: 2 mmol TEA and 20 ml CHMA were dissolved in DCM. Solution B: 0.2 mmol 4-arm PEG5000 was dissolved in 10 ml DCM. Solution A was added dropwise to Solution B under ice bath with gentle stirring for 30 min. Then put the mixture at room temperature to react for 24 h. After that, the mixture was dried by rotary evaporation and extracted by DCM three times. The resulting mixture was washed with ice water, hydrochloric acid (0.8 mM) and NaCl saturated solution three times separately to remove TEA. The organic layer was evaporated and the product was precipitated again using cold ethyl ether. Finally, 4-arm PEG_{5000}-CHMA was analyzed by 1H NMR (Bruker 600-MHz) and FT-IR (Bruker IFS 55). For the analyzation of 1H NMR, final material needs solute into $CDCl_3$. For the analyzation of FT-IR, the final material needs to be mixed with potassium bromide and press into tablet.

2.3. Preparation of PEGylated nanoemulsions

The oil phase containing TN, MCT, S75, $mPEG_{2000}$-DSPE and/or 4-arm PEG_{5000}-CHMA (Table 1) were dissolved under 55 °C. The water phase (sterile water for injection) which was kept in the same temperature was dropped into that oil phase quickly while stirring. Agitation was held for 10 min in 55 °C water bath to obtain the primary emulsions. The final emulsion was obtained by using a laboratory ultrasonic cell pulverizer (JY92-II, Ningbo Scientz Biotechnology. Zhejiang, China) at 200 W for 2 min and at 400 W for 6 min. The emulsions were sized by extrusion through polycarbonate membranes with a pore size measuring 0.22 μm. The final emulsion was adjusted to an isotonic level by using 50% (*m/v*) glucose solution. The particle size distribution was estimated by the dynamic light scattering method using the Submicron Particle Sizer (Nicomp 380™; Particle Sizing Systems, Inc., Santa Barbara CA, USA). Using the same method, the conventional nanoemulsions, which omit the $mPEG_{2000}$-DSPE and/or 4-arm PEG_{5000}-CHMA in the oil phase, was prepared.

The following are the abbreviations about PEGylated nanoemulsions: P_{10}NE-1 (2), the first (second) injection of nanoemulsions modified with 10 mol% $mPEG_{2000}$-DSPE; C_5NE-1 (2), the first (second) injection of nanoemulsions modified with

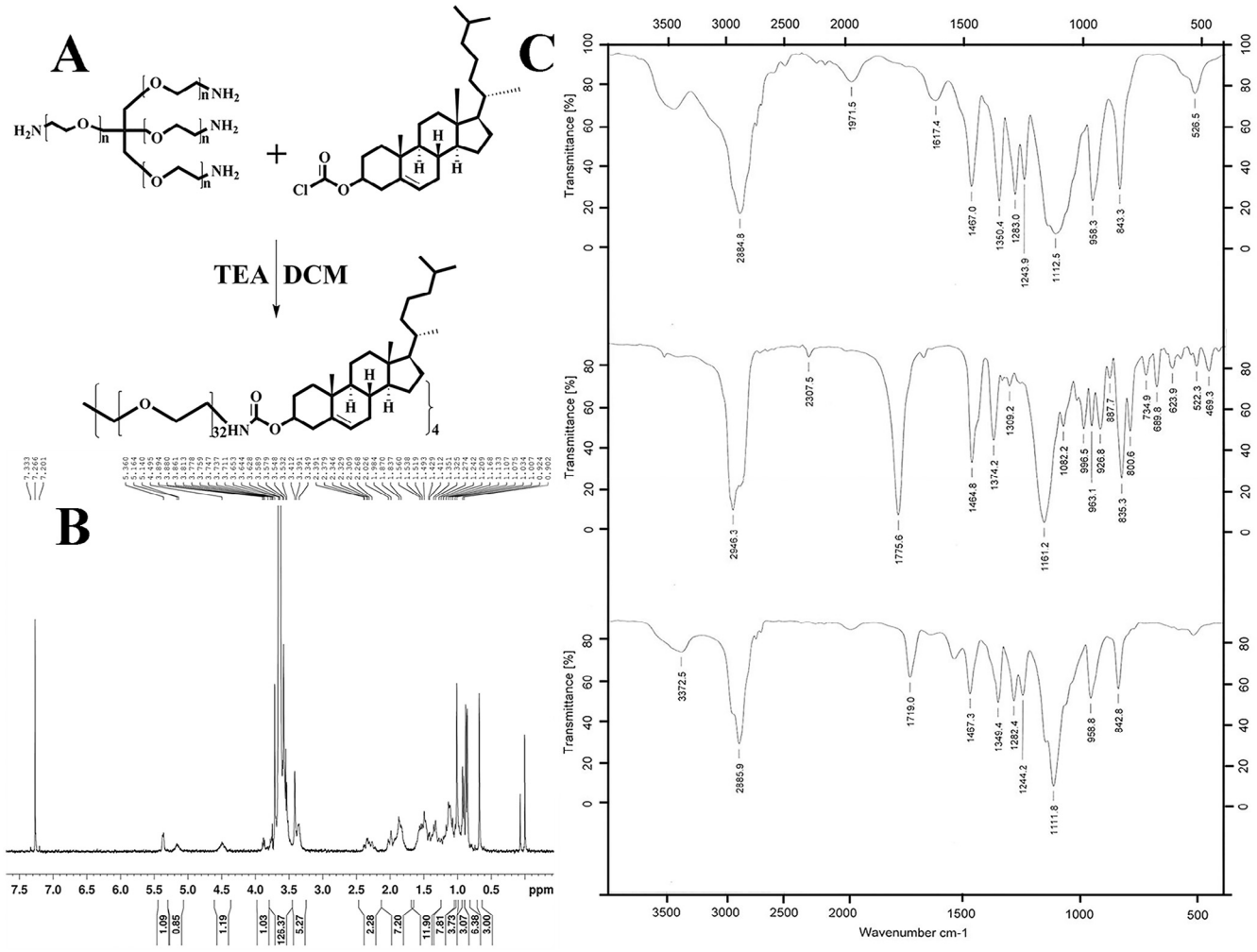

Fig. 1 – Synthesis and characterization of 4-arm PEG_{5000}-CHMA, n = 32. (A) Synthesis of 4-arm PEG_{5000}-CHMA. (B) ^1H NMR spectrum of 4-arm PEG_{5000}-CHAM. (C) FT-IR spectrum of PEG, CHMC, 4-arm PEG5000-CHMA.

5 mol% 4-arm PEG_{5000}-CHMA; C_{10}NE-1 (2), the first (second) injection of nanoemulsions modified with 10 mol% 4-arm PEG_{5000}-CHMA; C_5P_5NE-1 (2), the first (second) injection of nanoemulsions decorated with 5 mol% 4-arm PEG_{5000}-CHMA and 5 mol% $mPEG_{2000}$-DSPE; $P_{10}C_5$NE-1 (2), the first (second) injection of nanoemulsions decorated with 5 mol% 4-arm PEG_{5000}-CHMA and 10 mol% $mPEG_{2000}$-DSPE.

2.4. *Biodistribution and pharmacokinetics of PEGylated nanoemulsions*

The male Wistar rats weighing 180~200 g were purchased from the Experimental Animal Center of Shenyang Pharmaceutical University (Liaoning, China). All animal care and experiments were carried out according to the guidelines of the Animal Welfare Committee of Shenyang Pharmaceutical University. In our pre-stage work, we confirm that PEGlylated nanoemulsions would induce the strongest ABC phenomenon at a dose of 5 μmol phospholipid/kg which is larger than PEGylated liposome (0.001 μmol phospholipid/kg) (data never given here).

For the first injection, nanoemulsions we prepared, at doses of 5 μmol phospholipid/kg, were injected via the femoral vein. Control group received 5% (*m/v*) glucose solution instead of PEGylated nanoemulsions as following schemes (Table 2). For the second injection, corresponding nanoemulsions were injected intravenously via the femoral vein at a dose of 5 μmol

Table 1 – The composition of nanoemulsions.

Nanoemulsions	Composition
P_{10}NE	TN/MCT/S75/$mPEG_{2000}$-DSPE (2.2/10.8/2.5/1.0, *w/w/w/w*)
C_5NE	TN/MCT/S75/4-arm PEG_{5000}-CHMA (2.0/10.0/2.5/1.0, *w/w/w/w*)
C_{10}NE	TN/MCT/S75/4-arm PEG_{5000}-CHMA (1.0/5.0/1.2/1, *w/w/w/w*)
C_5P_5NE	TN/MCT/S75/4-arm PEG_{5000}-CHMA/$mPEG_{2000}$-DSPE (4.4/21.7/5.3/1.0/2.2, *w/w/w/w/w*)
$P_{10}C_5$NE	TN/MCT/S75/4-arm PEG_{5000}-CHMA/$mPEG_{2000}$-DSPE (2.4/11.9/2.7/1.0/1.1, *w/w/w/w/w*)
Conventional nanoemulsions	TN/MCT/S75 (1.0/5.0/2.1, *w/w/w*)

Table 2 – The injection scheme of the PEGylated nanoemulsions.

Group	The first treatment (5 μmol phospholipids/kg)	The second treatment (5 μmol phospholipids/kg)
1	5% glucose solution	Conventional emulsion
2	5% glucose solution	C_5NE
3	5% glucose solution	C_{10}NE
4	5% glucose solution	P_{10}NE
5	5% glucose solution	C_5P_5NE
6	5% glucose solution	C_5P_{10}NE
7	P_{10}NE	P_{10}NE
8	C_5P_5NE	C_5P_5NE
9	C_5P_{10}NE	C_5P_{10}NE

phospholipid/kg. The injection interval was 7 d. At selected post-injection time points (0.083, 0.25, 0.5, 1, 2 and 4 h), blood was sampled through eye marginal vein. The liver and spleen were removed 4 h after the last blood sample was withdrawn. The plasma samples and tissue samples were treated as follows: 100 μl of the plasma samples or homogenates (equivalent to 0.5 g tissue) were mixed with methanol (100 μl), an internal standard (100 μl), tocopheryl acetate (100 μg/ml) and n-hexane (600 μl). The entire mixture was vortexed for 5 min and centrifuged at 10,000 rpm for 10 min. The supernatant (500 μl) was dried using a CentriVap® Centrifugal Vacuum Concentrator (Labconco Corporation, Kansas City, USA) and dissolved in the mobile phase (100 μl). The resulting mixture was vortexed for 1 min and centrifuged at 10,000 rpm for 10 min. The supernatant (20 μl) was collected and used for analysis by high performance liquid chromatography (HPLC) method using a P230 pump and a UV230 UV/Vis Detector (Da Lian Elite Analytical Instruments Co., Ltd., China) composed of a Hypersil BDS C18 column (200 mm × 4.6 mm) containing particles measuring 5 μm in diameter at 30 °C. The ultraviolet wavelength was 264 nm. The mobile phase was methanol/isopropanol (80 : 20, υ/υ) at a flow rate of 1 ml/min.

2.5. *The calculation of ABC index*

In order to quantitatively evaluate the degree of this phenomenon, ABC index$_{(0-\infty)}$, a scientific and simple concept, was proposed. In our study, the ABC index in 4 h could stand for the extent of ABC phenomenon efficiently, because the second dose was almost cleared from the circulation in 4 h. The calculation function: ABC index$_{(0-4h)}$ = AUC$_{(0-4\ h)}$ of the second dose/AUC$_{(0-4\ h)}$ of the control dose. The ABC indexes of three preparations are presented in Table 3.

2.6. *ELISA for detecting the anti-PEG IgM*

Before the second injection, the blood samples were collected. The serum was gained after standing 2 h by centrifugation (4000 rpm, 10 min). The content of anti-PEG IgM in the serum was detected by ELISA [9]. Experimental procedure was as follows: 50 μl of mPEG$_{2000}$-DSPE ethanol solution was added (content 0.56 mg/ml mPEG$_{2000}$-DSPE) into a 96-well plate (USA, Corning), then dried under room temperature. The plate was blocked with 100 μl of Tris (USA, Sigma-Aldrich)-buffered saline (pH 8.0) containing 1% BSA (Bovine Serum Albumin, Korea, Biosharp). After incubating for 1 h at room temperature, the plate was washed three times with wash solution that is tris-buffered saline (pH 8.0) containing 0.05% Tween 20 (USA, Sigma-Aldrich). Serum samples was diluted (100 μl, 1:100, υ/υ) by the diluted solution (tris-buffered saline contained 1% of BSA and 0.05% of Tween 20, pH 8.0) and added to the 96 wells. After 1 h incubation, five times washing was required. Then horseradish peroxidase conjugated rabbit anti-rat IgM (China, Beijing Biosynthesis Biotechnology Co., Ltd) (100 μl, 1 μg/ml) was added into each well and incubated in room temperature for 1 h, then washed five times. 1 mg/ml of O-phenylendiamine (USA, Sigma-Aldrich) solution (solvent was Phosphoric-citric acid buffer, pH 5.0) was added into each well. After incubating for 15 min, the reaction was stopped by adding 100 μl of 1 mol/l H_2SO_4. The absorbance was measured at 490 nm and 630 nm by a microplate reader (UK, Bio-Rad Laboratories Ltd., Hertfordshire).

2.7. *Theoretical calculations*

The data are presented as the mean ± standard deviation. The statistical analysis was performed using Student's t-test with SPSS 16.0 (SPSS Inc., USA) software. The bond lengths and the bond angles are calculated by ChemBio3D Ultra 14.0 (CambridgeSoft Inc., USA).

Table 3 – The main pharmacokinetic parameters of the injected nanoemulsions in rats (n = 3).

Injected dose	AUC$_{(0-\infty)}$ (mg/l/h)	ABC index AUC$_{(0-4\ h)}$ (mg/l/h)	CL (l·h/kg)	T1/2z (h)	MRT$_{(0-\infty)}$ (h)	ABC index$_{(0-4\ h)}$
Conventional emulsion	16.179 ± 0.933	10.934 ± 1.040	1.236 ± 0.263	0.053 ± 0.002	0.057 ± 0.013	
C_5NE	30.681 ± 1.203	30.680 ± 2.983	0.652 ± 0.047	0.287 ± 0.001	0.315 ± 0.005	
C_{10}NE	108.094 ± 6.529	106.055 ± 7.973	0.185 ± 0.016	0.695 ± 0.033	0.899 ± 0.023	
P_{10}NE						0.16 ± 0.01
First injection	440.832 ± 9.770	230.347 ± 11.670	0.045 ± 0.004	3.588 ± 0.174	5.260 ± 0.094	
Second injection	37.471 ± 2.043	36.531 ± 3.508	0.534 ± 0.033	0.781 ± 0.081	0.977 ± 0.003	
C_5P_5NE						0.35 ± 0.06
First injection	416.342 ± 9.331	215.748 ± 10.334	0.048 ± 0.007	3.676 ± 0.142	5.363 ± 0.079	
Second injection	120.094 ± 8.748	74.933 ± 5.247	0.167 ± 0.052	3.223 ± 0.119	4.124 ± 0.082	
C_5P_{10}NE						0.61 ± 0.15
First injection	583.327 ± 13.579	220.228 ± 12.376	0.034 ± 0.001	5.996 ± 0.190	8.540 ± 0.130	
Second injection	248.795 ± 11.430	133.327 ± 7.893	0.080 ± 0.002	3.765 ± 0.163	5.245 ± 0.127	

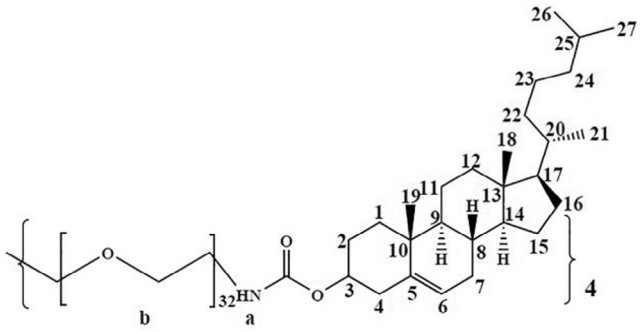

Fig. 2 – The atomic number of 4-arm PEG_{5000}-CHMA.

Table 4 – The characterization of nanoemulsions.

Formulations	Mean diameter (nm)	C.V.	*Zeta* potential (mV)
Conventional emulsions	230.2 ± 6.1	0.318 ± 0.012	−15.50 ± 3.90
$P_{10}NE$	119.4 ± 4.2	0.388 ± 0.007	−30.82 ± 1.77
C_5NE	207.4 ± 5.7	0.374 ± 0.008	−7.59 ± 1.37
$C_{10}NE$	124.2 ± 4.8	0.330 ± 0.013	−5.64 ± 3.55
C_5P_5NE	135.2 ± 3.2	0.428 ± 0.011	−17.54 ± 5.22
$C_5P_{10}NE$	113.6 ± 6.6	0.390 ± 0.008	−29.67 ± 1.09

3. Results and discussion

3.1. *The structure confirmation of 4-arm PEG_{5000}-CHMA*

The structure of 4-arm PEG_{5000}-CHMA was confirmed by ^{1}H-NMR and IR, the results are shown in Fig. 1. The atomic numbers of 4-arm PEG_{5000}-CHMA are shown in Fig. 2. ^{1}H-NMR ($CDCl_3$, δ ppm): 7.266 is the solvent peak of $CDCl_3$; 0.675 (s, 3H, H-18); 0.854, 0.873 (d, 6H, H-26, 27); 0.902, 0.924 (d, 3H, H-21); 1.007 (s, 3H, H-21); 2.338 (br.d, 2H, H-4); 3.644 (m, 126H, H-b); 5.164 (m, H, H-a); 5.360 (m, 1H, H-6). In ^{1}H-NMR spectrum, a broad peak in δ 3.644 ppm is the most obvious characteristic signal which stands for -$(CH_2CH_2O)_{32}$-. The rest of each peak is similar with the ^{1}H-NMR ($CDCl_3$) message of cholesterol methyl chloride.

In addition, the connection of 4-arm PEG_{5000} to CHMA was also verified with FT-IR spectroscopy by the presence of carbonyl stretching bands (at around 1719 cm^{-1}) in amino linkage and the carbonyl stretching vibration peak (at 1775 cm^{-1}) of CHMA disappeared. Each arm of 4-arm PEG_{5000} links with one CHMA. We set the peak integral of methyl protons (12 altogether) in all four cholesterol as 3, therefore the integral for broad peak which presents four $(CH2CH2O)_{32}$ groups of PEG chain in 4-arm PEG_{5000}-CHMA could be 126. The other peaks are similar to CHMA. These results indicate that the 4-arm PEG_{5000}-CHMA has been successfully synthesized. Then we prepared nanoemulsions using the 4-arm PEG_{5000}-CHMA or/and $mPEG_{2000}$-DSPE (Fig. 3).

3.2. *The characterization of nanoemulsions*

The mean diameters of nanoemulsions are range from 120 nm to 230 nm (Table 4). Moreover, the coefficient of variation (C.V.) value of all the formulations which expressed the particle size distribution ranges from 0.318 to 0.428. Table 4 also reveals that all nanoemulsions are negatively charged because under physiological pH values, one $mPEG_{2000}$-DSPE and one S75 molecule carries one negative charge while 4-arm PEG_{5000}-CHMA is electric neutrality. Generally, nanoemulsions which carry more $mPEG_{2000}$-DSPE polymer are more negatively charged and the shield by 4-arm PEG5000 on the surface of nanoemulsions neutralizes the zeta-potential of nanoemulsions. Finally, we confirm that $mPEG_{2000}$-DSPE and 4-arm PEG_{5000}-CHMA can insert into the surface of nanoemulsions.

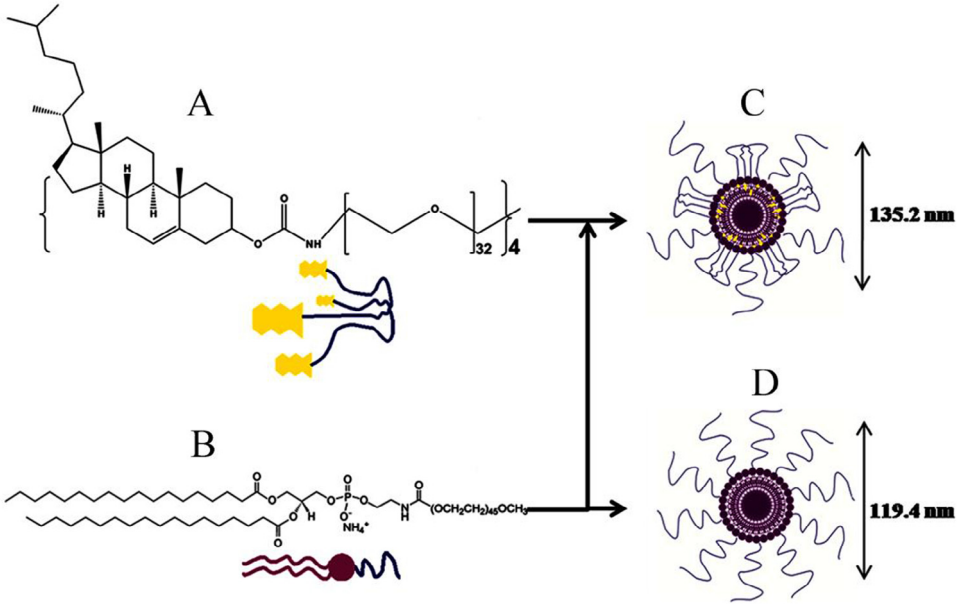

Fig. 3 – (A, B) The structural chemical formulae of the 4-arm PEG_{5000}-CHMA and $mPEG_{2000}$-DSPE. (C, D) Schematic presentation of the CPNEs and PNEs.

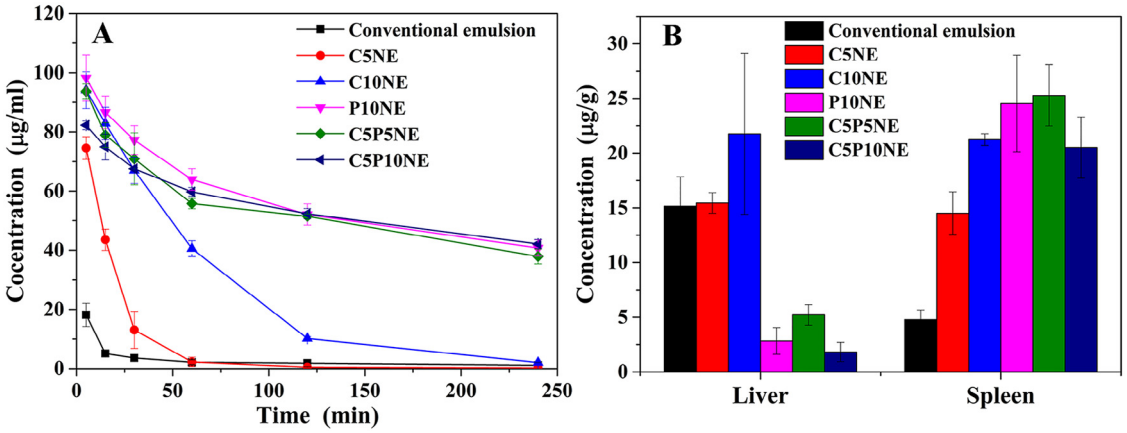

Fig. 4 – (A) The blood clearance profile of PEGylated nanoemulsions in rats. Rats were administrated with PEGylated emulsions at a dose of 5 μmol phospholipids/kg. Data are shown as mean ± SD, n = 3. (B) Hepatic and splenic accumulations 4 h after *i.v.* injection of the test dose of PEGylated nanoemulsions. Data are shown as mean ± SD, n = 3.

3.3. *Pharmacokinetics of the PEGylated nanoemulsions in rats*

When we prepared nanoemulsions coated with 4-arm PEG_{5000}-CHMA only (CNE), the lifetime ($MRT_{(0-\infty)}$, 0.315 ± 0.005 h (C_5NE), 0.899 ± 0.023 h ($C_{10}NE$)) in circulation is longer than that of the conventional emulsions ($MRT_{(0-\infty)}$, 0.057 ± 0.013 h), especially before 1 h after the injection (Fig. 4). But, compared with nanoemulsions coated with $mPEG_{2000}$-DSPE only (PNE), the CNE cannot keep an relatively high level in circulation like PNE ($MRT_{(0-\infty)}$, 5.260 ± 0.094 h ($P_{10}NE$)). That means the 4-arm PEG_{5000}-CHMA cannot perfectly protect the nanoemulsions in a persistent state. When modifying the nanoemulsions with the 4-arm PEG_{5000}-CHMA and PEG_{2000}-DSPE, the mixed PEGylated surfactant modifying system, the circulation time of nanoemulsions was prolonged by forming an stable hydrated layer ($MRT_{(0-\infty)}$, 5.363 ± 0.079 h (C_5P_5NE), 8.540 ± 0.130 h ($C_5P_{10}NE$)). Hence, the circulation time of $P_{10}NE$,C_5P_5NE,$C_5P_{10}NE$ was further prolonged. Moreover, It has also been proposed that the ABC phenomenon have association with circulation time [16,18]. Hence, prepare the different polymer modify formulations in similar level in circulation time is also basic to compare the decrease extent of ABC phenomenon scientifically.

3.4. *ABC phenomenon of the PEGylated nanoemulsions in rats*

Over the past decades, several creative approaches have been reported to circumvent the ABC phenomenon, such as changing the physicochemical properties, ameliorating the administration regimen, or finding other alternative polymers.

Changing the physicochemical properties: PEGyalted nanoparticles in optimal PEG modify density elicit a maximal ABC phenomenon, while, at either lower or higher surface density of PEG, the ABC phenomenon is reduced. The optimal PEG modify density value will change a lot between different type of nanoparticles. For example, Ishida reported the optimal PEG modify density of PEGylated liposome is 5 mol% [19]. Zhao demonstrated that solid lipid nanoparticles (SLNs) containing 10 mol% PEG produced a higher elimination rate [10]. In addition, PEGylated nanoparticles in lower size can decrease even evade the ABC phenomenon. Koide demonstrated that the ABC phenomenon cannot be induced in the profile of repeated injection of PEG-pAsp(pentyl) micelles (33.6 nm) [20]. Kaminskas reported repeated injected PEG_{2000}-DSPE micelles (18 nm) also cannot induce the ABC phenomenon [21]. Based on these experiences, we can choose the most reasonable type of nanoparticle for clinical use. Obviously, these methods are not enough to solve the ABC phenomenon of various nanoparticles.

Ameliorating the administration regimen: The ABC phenomenon can be circumvented by increasing the injection dose for the nanoparticles which modified with materials that have a higher toxicity dose for intravenous injection. As reported, with intravenous injection of 150 mg Lactosome/kg into rat, no ABC phenomenon was found [22]. Similarly, the liposome modified with PEGylated hemoglobin (Hb), at the dose of 1400 mg Hb/kg, ABC phenomenon disappeared [23]. In addition, inducing the strongest intensity of ABC phenomenon also needs an appropriate time interval between the first and the second injection, such as the optimal time interval of mice [24], rat [25], and beagle dogs [10] was 10, 5, and 7 d. When changing the injection interval, the ABC phenomenon can be decreased. Moreover, micelles used alternatively with liposome can also circumvent the ABC phenomenon [21]. Those methods could also circumvent a part of nanoparticles' ABC phenomenon. However, we need to rethink about the influence of the curative effect by ameliorating the administration regimen.

Finding other alternative polymers: As mentioned earlier, poly(N-vinyl-2-pyrrolidone), poly(4-acryloylmorpholine), or poly(N,N-dimethylacrylamide) and polyglycerol (PG) can decrease the ABC phenomenon, although scarify a certain degree of circulation time [14,15]. In 2015, Li reported that Poly(carboxybetaine) can be a new material that do not induce the ABC phenomenon, but the article never study the ABC phenomenon under different injection dose [26]. In 2016, Zhang studied that hyaluronic acid can also circumvent the ABC phenomenon at the dose of 0.1 and 5 μmol lipid/kg [27]. These new materials all made important progress in the area of eliminating the ABC phenomenon. It is the most difficult but highly effective method to solve these problems. Reviewing the progress

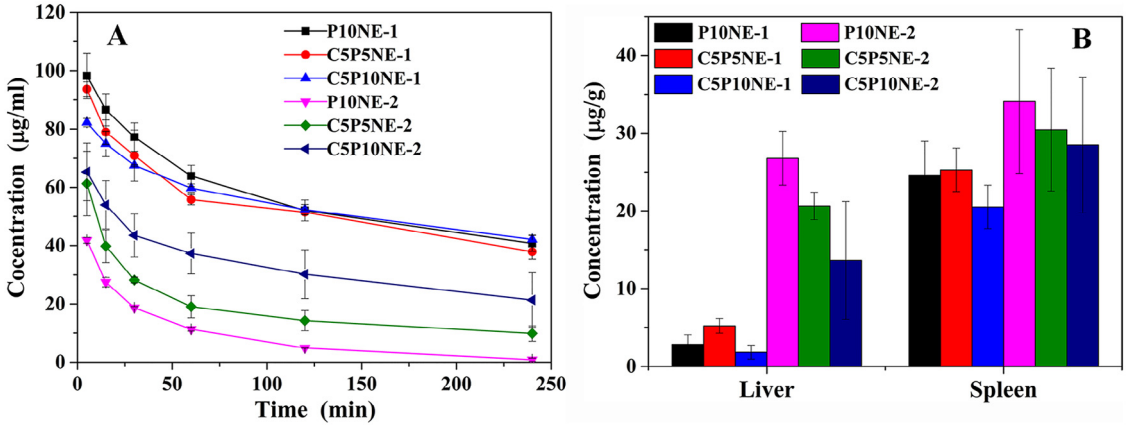

Fig. 5 – (A) The blood clearance profile of $P_{10}NE$, C_5P_5NE and $C_5P_{10}NE$ in rats. Rats were pretreated with $P_{10}NE$, C_5P_5NE and $C_5P_{10}NE$ (5 μmol phospholipid/kg) or 5% glucose solution, at 7 d post-first injection, all groups were repeated administrated with $P_{10}NE$, C_5P_5NE and $C_5P_{10}NE$ separately at a dose of 5 μmol phospholipids/kg. Data are shown as mean ± SD, n = 3. (B) Hepatic and splenic accumulations 4 h after i.v. injection of the test dose. Data are shown as mean ± SD, n = 3.

we had achieved, we found that a mixed modifier method, which also had the chance to decrease or even remove the ABC phenomenon, was never studied at all. Our work aimed to give an idea that the materials we had can give consideration of both sides; lower immunology and excellent stealth characters, by combining with other modifier methods.

We chose $P_{10}NE$, C_5P_5NE and $C_5P_{10}NE$ which are in the similar level in circulation lifetime to study whether 4-arm PEG_{5000}-DSPE is helpful to weaken the ABC phenomenon. Rats were treated with $P_{10}NE$, C_5P_5NE and $C_5P_{10}NE$ (5 μmol phospholipids/kg) as the first dose. Set the dose interval as 7 d, the rats were injected with $P_{10}NE$, C_5P_5NE and $C_5P_{10}NE$ (5 μmol phospholipids/kg) as the second dose. Table 1 shows the treatment schemes. As expected, repeated injection with $P_{10}NE$, triggered the rapid clearance of the second dose of $P_{10}NE$ from circulation (Fig. 5A) and increased uptake by the liver and spleen (Fig. 5B). ($P_{10}NE$: $ABC_{index\ (0\text{–}4\ h)} = 0.16 \pm 0.01$). But from Table 3, the ABC phenomenon is further decreased by CPNEs (C_5P_5NE: $ABC_{index\ (0\text{–}4\ h)} = 0.35 \pm 0.06$; $C_5P_{10}NE$: $ABC_{index\ (0\text{–}4\ h)} = 0.61 \pm 0.15$). In addition, although the nanoemulsions in the similar circulation time, the ABC phenomenon induced was in different extent ($P_{10}NE > C_5P_5NE > C_5P_{10}NE$).

3.5. *Production of anti-PEG IgM*

Previous studies suggest that anti-PEG IgM production levels have a positive correlation with ABC phenomenon. ELISA was used to detect the anti-PEG IgM levels. In this study, ELISA was also used to study the combination ability of anti-PEG IgM to different antigens using a 96 wells plates coated with two antigens (4-arm PEG_{5000}-CHMA or $mPEG_{2000}$-DSPE). The antibody combination of anti-PEG IgM is important for following the complement activation and the mononuclear phagocyte system uptake. As shown in Fig. 6, PEGylated nanoemulsions induced the production of substantial amounts of anti-PEG IgM. The order of antibody level is, $C_5P_5NE > P_{10}NE > C_5P_{10}NE$. Interestingly, the extent of the ABC phenomenon is not completely consistent with antibody level. Moreover, the antibody was easier to combine with $mPEG_{2000}$-DSPE than 4-arm PEG_{5000}-CHMA group for the higher OD value in the covered $mPEG_{2000}$-DSPE group than the covered 4-arm PEG_{5000}-CHMA group. That means except antibody level, antibody combination with modify polymers also play an important role in the induction of the ABC phenomenon. When the antibody in a similar level such as C_5P_5NE, $P_{10}NE$, the antibody combination has a relationship with the extent of the ABC phenomenon. Because the antibody prefers to combine with the PEG_{2000}-DSPE, the ABC phenomenon of $P_{10}NE$ was stronger than C_5P_5NE group. In this study, we proposed covering up all the terminal group of 4-arm PEG_{5000} with cholesterol having the chance to circumvent the ABC phenomenon by decreasing the antibody binding.

As reported [28], star shaped PEG chains, which provide a steric stabilization, reduce complement activation of nanoparticles. Hence, although the antibody produced by C_5P_5NE is more than $P_{10}NE$ group, the ABC phenomenon of $P_{10}NE$ is stronger than C_5P_5NE group for the reason of having a higher level of complement activation. In addition, the complement activation needs the antibody IgM stay in mushroom conformation [29]. We assumed that the conformations of surface layer of emulsions become complicated in the condition of blending different structure of PEG in the formulation. Because of the relatively complicated transformation in conformation, the recognition of anti-PEG antibody would be difficult to stay mushroom conformation on the surface of nanoemulsions. Then the complement activation decreased. Therefore, we considered that the antibody level and following antibody combination are both important for the induction of ABC phenomenon. That is to say, mixed PEGylated surfactant modifying system can decrease the ABC phenomenon and will direct more promising colloidal drug carriers in the future.

4. Conclusion

In summary, a novel material, 4-arm PEG_{5000}-CHMA, has been synthesized successfully and the nanoemulsions modified with 4-arm PEG_{5000}-CHMA and/or $mPEG_{2000}$-DSPE has been prepared.

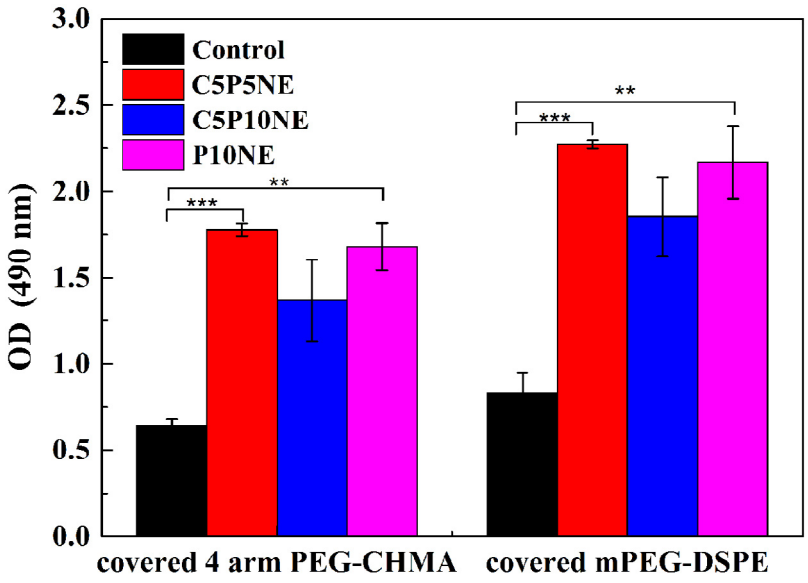

Fig. 6 – The anti-PEG IgM production following intravenous injections of Ragged-NEs. Black square means the serum obtained from the rat without control; other squares mean the serum obtained from the rat pretreated with C_5P_5NE,$C_5P_{10}NE$ and $P_{10}NE$. Each value represents the mean ± SD (n = 3). *P* values apply to differences between the control and treated rats.*P < 0.05, **P < 0.01, ***P < 0.001.

Furthermore, the pharmacokinetic character and the ABC phenomenon of PEGylated nanoemulsions have been detailed investigated. Our researches suggest that the circulation time is prolonged and the ABC phenomenon can be decreased at the same time by nanoemulsions modified with 4-arm PEG_{5000}-CHMA and $mPEG_{2000}$-DSPE. We propose that the ABC phenomenon is decreased by the antibody secretion and the further antibody combination. In this work, mixed PEGylated surfactant modifying system can reach the aim of the decrease of ABC phenomenon as well as an ideal pharmacokinetic character. Thus, nanoemulsions with 4-arm PEG_{5000}-CHMA and $mPEG_{2000}$-DSPE have chance to be a promising nanocarrier for clinical use.

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REFERENCES

[1] Dams ET, Laverman P, Oyen WJ, et al. Accelerated blood clearance and altered biodistribution of repeated injections of sterically stabilized liposomes. J Pharmacol Exp Ther 2000;292:107–1079.
[2] Ishida T, Maeda R, Ichihara M, et al. Accelerated clearance of PEGylated liposomes in rats after repeated injections. J Control Release 2003;88:35–42.
[3] Ishida T, Masuda K, Ichikawa T, et al. Accelerated clearance of a second injection of PEGylated liposomes in mice. Int J Pharm 2003;255:167–174.
[4] Ishida T, Atobe K, Wang X, et al. Accelerated blood clearance of PEGylated liposomes upon repeated injections: effect of doxorubicin-encapsulation and high-dose first injection. J Control Release 2006;115:251–258.
[5] Xu H, Ye F, Hu M, et al. Influence of phospholipid types and animal models on the accelerated blood clearance phenomenon of PEGylated liposomes upon repeated injection. Drug Deliv 2015;22:598–607.
[6] Ishida T, Kashima S, Kiwada H. The contribution of phagocytic activity of liver macrophages to the accelerated blood clearance (ABC) phenomenon of PEGylated liposomes in rats. J Control Release 2008;126:162–165.
[7] Ishida T, Wang X, Shimizu T, et al. PEGylated liposomes elicit an anti-PEG IgM response in a T cell-independent manner. J Control Release 2007;122:349–355.
[8] Wang X, Ishida T, Kiwada H. Anti-PEG IgM elicited by injection of liposomes is involved in the enhanced blood clearance of a subsequent dose of PEGylated liposomes. J Control Release 2007;119:236–244.
[9] Wang L, Wang C, Jiao J, et al. Tolerance-like innate immunity and spleen injury: a novel discovery via the weekly administrations and consecutive injections of PEGylated emulsions. Int J Nanomedicine 2014;9:3645–3657.
[10] Zhao Y, Wang L, Yan M, et al. Repeated injection of PEGylated solid lipid nanoparticles induces accelerated blood clearance in mice and beagles. Int J Nanomedicine 2012;7:2891–2900.
[11] Koide H, Asai T, Kato H, et al. Size-dependent induction of accelerated blood clearance phenomenon by repeated injections of polymeric micelles. Int J Pharm 2012;432:75–79.
[12] Shimizu T, Ichihara M, Yoshioka Y, et al. Intravenous administration of polyethylene glycol-coated (PEGylated) proteins and PEGylated adenovirus elicits an anti-PEG immunoglobulin M response. Biol Pharm Bull 2012;35:1336–1342.
[13] Yang Q, Ma Y, Zhao Y, et al. Accelerated drug release and clearance of PEGylated epirubicin liposomes following

repeated injections: a new challenge for sequential low-dose chemotherapy. Int J Nanomedicine 2013;8:1257–1268.
[14] Ishihara T, Maeda T, Sakamoto H, et al. Evasion of the Accelerated Blood Clearance Phenomenon by coating of nanoparticles with various hydrophilic polymers. Biomacromolecules 2010;11:2700–2706.
[15] Abu Lila AS, Nawata K, Shimizu T, et al. Use of polyglycerol (PG), instead of polyethylene glycol (PEG), prevents induction of the accelerated blood clearance phenomenon against long-circulating liposomes upon repeated administration. Int J Pharm 2013;456:235–242.
[16] Saadati R, Dadashzadeh S, Abbasian Z, et al. Accelerated blood clearance of PEGylated PLGA nanoparticles following repeated injections: effects of polymer dose, PEG coating, and encapsulated anticancer drug. Pharm Res 2013;30:985–995.
[17] Maeda H, Wu J, Sawa T, et al. Tumor vascular permeability and the EPR effect in macromolecular therapeutics. J Control Release 2000;65:271–284.
[18] Xu H, Wang KQ, Deng YH, et al. Effects of cleavable PEG-cholesterol derivatives on the accelerated blood clearance of PEGylated liposomes. Biomaterials 2010;31:4757–4763.
[19] Ishida T, Harada M, Wang XY, et al. Accelerated blood clearance of PEGylated liposomes following preceding liposome injection: effects of lipid dose and PEG surface-density and chain length of the first-dose liposomes. J Control Release 2005;105:305–317.
[20] Koide H, Asai T, Hatanaka K, et al. Particle size-dependent triggering of accelerated blood clearance phenomenon. Int J Pharm 2008;362:197–200.
[21] Kaminskas LM, McLeod VM, Porter CJ, et al. Differences in colloidal structure of PEGylated nanomaterials dictate the likelihood of accelerated blood clearance. J Pharm Sci 2011;100:5069–5077.
[22] Hara E, Makino A, Kurihara K, et al. Evasion from accelerated blood clearance of nanocarrier named as “Lactosome” induced by excessive administration of Lactosome. Biochim Biophys Acta 2013;1830:4046–4052.
[23] Taguchi K, Urata Y, Anraku M, et al. Hemoglobin vesicles, polyethylene glycol (PEG)ylated liposomes developed as a red blood cell substitute, do not induce the accelerated blood clearance phenomenon in mice. Drug Metab Dispos 2009;37:2197–2203.
[24] Ishida T, Ichikawa T, Ichihara M, et al. Effect of the physicochemical properties of initially injected liposomes on the clearance of subsequently injected PEGylated liposomes in mice. J Control Release 2004;95:403–412.
[25] Ishida T1, Maeda R, Ichihara M, et al. Accelerated clearance of PEGylated liposomes in rats after repeated injections. J Control Release 2003;88:35–42.
[26] Li Y, Liu R, Shi Y, et al. Zwitterionic poly (carboxybetaine)-based cationic liposomes for effective delivery of small interfering RNA therapeutics without accelerated blood clearance phenomenon. Theranostics 2015;5:583–596.
[27] Zhang Q, Deng C, Fu Y, et al. Repeated administration of hyaluronic acid coated liposomes with improved pharmacokinetics and reduced immune response. Mol Pharm 2016;doi:10.1021/acs.molpharmaceut.5b00952.
[28] Mosqueira VC, Legrand P, Gulik A, et al. Relationship between complement activation, cellular uptake and surface physicochemical aspects of novel PEG-modified nanocapsules. Biomaterials 2001;22:2967–2979.
[29] Czajkowsky DM, Shao Z. The human IgM pentamer is a mushroom-shaped molecule with a flexural bias. Proc Natl Acad Sci USA 2009;106:14960–14965.